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Research paper

Effect of surfactant on 5-aminolevulinic acid uptake and PpIX generation in human cholangiocarcinoma cell

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ABSTRACT

Photodynamic therapy (PDT) is a palliative therapy and has been used to cure cholangiocarcinoma (CC), which has a poor prognosis and limited available curative therapy. PDT was shown to improve the median survival time of advanced-stage patients. Recently, 5-aminolevulinic acid (ALA) has been used as a pro-photosensitizer, which can be transferred to intercellular protoporphyrin IX (PpIX), which is a strong photosensitizer, via the heme pathway. The main limitation of using ALA in PDT is the hydrophilic properties of ALA, which results in low cellular uptake. In this study, non-ionic surfactants, pluronic F68 (PF68) and Tween 80 (TW80), were used to address this limitation. The human CC cell line, HuCC-T1, was cotreated with ALA and different concentrations of surfactants for 4 h. The effect of surfactants was evaluated by monitoring the uptake of ALA, the fluorescence intensity of PpIX, and the cell survival rate after suitable light irradiation. Cotreatment with the surfactant resulted in an increased intracellular ALA level, PpIX formation, and phototoxicity.

1. Introduction

Cholangiocarcinoma (CC) is an aggressive cancer arising from the malignant transformation of cholangiocytes, which are the epithelial cells lining the biliary tree. CC prognosis is grim due to a lack of viable treatment options, and the median survival of advanced-stage CC is less than 24 months [1,2]. The only curative therapy of CC is surgical resection, but unfortunately, the majority of patients definitely diagnosed with CC are already in the advanced stage, where surgical treatment is no longer an option. Therefore, palliative therapies are quite important to the management of CC, mainly including endoscopic stent placement, chemotherapeutic, radiation therapy, and photodynamic therapy (PDT).

Recently, PDT was used as a palliative therapy for the treatment of CC with only minimal side effects for the patient. In PDT, two non-toxic components, a photosensitizer and light, are applied in sequence and the PDT can induce cancer cell apoptosis or necrosis with light following the administration of light-activated photosensitizing drugs. 5-Aminolevulinic acid (ALA) has gained enormous interest as an effective endogenous photosensitizer for fluorescence diagnosis and PDT [3]. Excessive administration of ALA can lead to successful PDT. ALA is well known as a precursor of a strong photosensitizing agent, protoporphyrin IX (PpIX) [4]. Under physiological conditions, ALA can be metabolized into PpIX via the heme biosynthetic pathway. PDT is carried out using red light, and then activated PpIX can lead to the production of cytotoxic reactive singlet oxygen species.

The main drawback of ALA-based PDT for clinical applications is the hydrophilic properties of ALA, which results in relatively low cellular uptake [5]. Many approaches have been developed to overcome this disadvantage. Esterification of ALA using aliphatic alcohols was conducted to improve the lipophilicity and increase the cellular uptake of ALA. In some case, ester-ALA improved cell membrane permeability and a good conversion rate to protoporphyrin than the parent compound was achieved [6,7]. Perotti et al. reported porphyrin synthesis from ALA-hexyl ester (He-ALA) [8]. In this case, the amount of interconverted PpIX was higher than that obtained for ALA at low concentrations, which was probably due to the existence of different uptake mechanisms for ALA and He-ALA by tumor cells. Therefore, some ester-ALA drugs, such as ALA-methyl ester or He-ALA, are considered potential alternative drugs to natural ALA. In addition, nanoparticles have also investigated as carriers for photosensitizers in PDT because nanocolloid lotion was shown to increase the ALA stability [9].

Another possible approach to increase the cellular uptake of ALA is the use of enhancers. Penetration enhancers, such as sulfoxide, alcohols, and surfactants, have been extensively investigated as delivery vehicles to penetrate the skin and mucous barrier [10]. Malik et al. reported that dimethylsulfoxide (DMSO)

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effectively improved the accumulation of PpIX in the skin and tumors of mice [11]. Casas et al. found that higher porphyrin accumulation was observed in the skin overlying tumor tissue when transplantable murine adenocarcinoma was treated with DMSO and ALA [12]. Also, Perotti et al. reported that the addition of DMSO to ALA formulations slightly increased porphyrin biosynthesis in the tumor and skin overlying the tumor [13]. Morrow et al. reported that several chemical penetration enhancers (such as DMSO and Labrafac CC) may have a greater effect on the delivery of lipophilic ALA prodrugs, but they did not improve the delivery of ALA [14]. Therefore, penetration enhancers hold promise for use in delivering ALA across skin or mucous barriers.

CC develops deeper into the skin, which severely limits the use of chemotherapy and radiotherapy for treatment. As a result, endoscopic operations are usually used to treat CC, and it can allow for ALA-based PDT of CC. The prodrugs and suitable light source can be delivered using an endoscope [15]. In addition, local application of ALA-based PDT through the endoscopic operation limits the frequency of undesirable side effects of the photosensitizers when compared with systemic application.

In a previous study, we studied the effect of ALA-based PDT against human cholangiocarcinoma HuCC-T1 cells, and successful PDT effects were observed when drug doses and incubation times were increased [16]. Here, we used penetration enhancers such as Tween 80 (TW80) and pluronic F68 (PF68) to increase ALA permeation in human cholangiocarcinoma cells. These kinds of surfactants are generally known to have low chronic toxicity and can enhance the flux of materials permeating through biological membranes [10]. The human cholangiocarcinoma cell line, HuCC-T1, was treated with ALA, and different concentrations of non-ionic surfactants and the intracellular levels of ALA, interconverted PpIX concentration, and phototoxicity after PDT were investigated.

2. Materials and methods

2.1. Cell culture

HuCC-T1 cells were cultured in RPMI 1640 medium (GIBCO) (supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotics) at 37 °C in 5% CO_2. The cells were subcultured twice a week.

2.2. Chemicals

ALA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide, and pluronic F68 were obtained from Sigma (Sigma Chem. Co, St Louis, MO, USA). Tween 80 was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.3. Analysis of 5-ALA by HPLC after derivatization

In all cell culture experiments, the ALA stock solution was freshly prepared with or without surfactants at a ALA concentration of 1 M. ALA uptake into the cells was determined after conversion to its fluorescent derivative by reacting with acetylacetone and formaldehyde. The resulting derivative was assayed by high-performance liquid chromatography (HPLC, Flexar LC, Perkin Elmer) as described by Oishi et al. with slight modifications [17]. Briefly, the cells were washed with PBS 2 times and used for ALA quantification. After cells were solubilized in lysis buffer (GenDEPOT, USA), the samples were centrifuged at 12,000 rpm for 5 min and the supernatant was used for analysis. The fluorescence derivatization of 5-ALA was performed as follows: 0.7 ml of the acetylacetone reagent (15:10:75, acetylacetone/ethanol/distilled water) were mixed with 0.09 ml of 10% formaldehyde and 0.01 ml sample. This solution was then vigorously shaken for 5 s and then heated to 100 °C with moderate shaking, followed by cooling for 10 min. A 10 μl of the ALA derivative was injected onto a Brwonlee C18 reversed-phase column (5 μm, 150 × 4.6 mm). Elution was performed at 40 °C using a mobile phase that consisted of methanol–water–acetic acid (49.5:49.5:1 v/v/v) at a flow rate of 1.0 ml/min. The fluorescence intensity of the eluate was monitored at an excitation/emission wavelength of 370/460 nm (Flexar Fluorescence LC Detectir, Perkin Elmer). The detection limit of this method was approximately 50 ng/ml.

2.4. Cytotoxicity assay

Cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h in 5% CO_2 at 37 °C. For starvation, the cells were then incubated in serum free RPMI media for 24h. After removing the culture medium, the wells were washed with PBS. Each well was then replaced with 100 μl of fresh RPMI media containing ALA or surfactants. The concentration of ALA and surfactants was up to 10.0 mM and 1 mg/ml, respectively. The cells were then incubated in the dark at 37 °C for 6 h. After that, the cells were washed with PBS 3 times, and the cytotoxicity was measured using the MTT assay.

2.5. PpIX production and quantification

Cells in 96-well plates were incubated with 100 μl of serum-free medium containing different concentration of ALA with or without surfactant. The samples were then washed with PBS 3 times and used for PpIX quantification. Briefly, cells were solubilized in lysis buffer (GenDEPOT, USA) and subjected to quantitative spectrofluorometry. The fluorescence signal from each well was measured using Tecan M200 fluorescence spectrometer at an excitation/emission wavelength of 485/635 nm. The PpIX value was compensated by protein.

2.6. Photodynamic treatment

Cells seeded in 96-well plate were cultured for 24 h. The cells were then washed with PBS, and 100 μl of serum-free medium containing ALA with or without various concentrations of surfactants was added to each well, followed by incubation for 4 h. The negative control was treated with serum-free medium in the absence of both ALA and surfactant, and treatment with ALA alone was used as a positive control. These plates were exposed to an expanded homogenous beam of 635 nm radiation (LED lamp, SH system) at a dose of 0.3–1.0 J/cm^2 as measured by a photo-radiometer (DeltaOhm HD2102-1, Italy). Immediately after irradiation, the medium was removed and washed with PBS. A 100 μl of fresh RPMI containing 10% FBS was then added, and the cells were incubated for an additional 24 h. Cell phototoxicity was determined using the MTT assay.

2.7. MTT assay

The PDT effect on cell proliferation was measured using a modified MTT assay. The MTT assay is based on the fact that live cells are able to cleave the tetrazolium ring to a molecule that absorbs at 570 nm in active mitochondria [18]. A 2×10^4 cells were cultured in 96-well plates. After removing the culture media, 100 μl of fresh medium with 25 μl of the MTT reagent (2 mg/ml in PBS) (Sigma, Chemical Co, St Louis, MO, USA) was added to each well. The cells were incubated at 37 °C for an additional 3 h. The cells were then lyzed with 100 μl of lysis buffer solution (10% sodium dodecyl sulfate in 0.01 N HCl) for 18 h, and absorbance was measured at 570 nm.

2.8. In vitro photosensitizing efficacy using FACScan analysis

To evaluate necrotic cells following photodynamic therapy (PDT), propidium iodide (PI) uptake was assessed using FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, CA). HuCC-T1 cells were treated with 0.25 mM ALA and surfactants for 4 h and were then exposed to a light dose of 1.0 J/cm^2. Cells were harvested by trypsinization and resuspended in PBS. The harvested cells were stained with PI (10 μg/ml). The cells were further incubated for 10 min in the dark and immediately analyzed using a FACScan flow cytometer with a 488 nm excitation laser line. The PI fluorescence was collected using a 575 ± 15 band-pass filter.

2.9. Quantification of total protein

Total protein content from each sample was determined using the Pierce BCA protein assay kit (Rockford, IL, USA) following the manufacturer's instructions. A series of dilutions of known concentration were prepared from bovine serum albumin (BSA) following the dilution scheme described in the standard test tube protocol. Briefly, 10 μl of the sample lysate was added to the 200 μl of reagents A and B (A/B = 50:1). After 30 min of incubation in a CO_2 incubator, colorimetric detection was performed using the Tecan M200 spectrometer at a wavelength of 562 nm. The protein concentration of the samples was determined based on the standard curve.

2.10. Statistical data analysis

Results are expressed as the means of at least three parallel experiments ± SD. Statistical data analysis was performed using the t test with $p < 0.05$ at the minimal level of significance.

3. Results

3.1. Cytotoxicity of prodrug and surfactants

The cytotoxicity of ALA against HuCCT-1 cells was examined. HuCCT-1 cells were exposed to various concentrations of ALA for 6 h in the dark, and the survivability of tumor cells was analyzed using the MTT assay. As shown in Fig. 1, the toxicity of treatment with 1.0 mM ALA was negligible, while cell survival slightly decreased at ALA concentration higher than 2.0 mM.

The toxicity of the surfactants against HuCC-T1 cells was also assessed. As shown in Fig. 2, the cytotoxicity of both TW80 and PF68 was negligible at 1 mg/ml, i.e., higher than 99% of the cells survived at 1 mg/ml of surfactant.

3.2. PpIX formation in tumor cells

Conversion of ALA to PpIX was examined over 30 h after the HuCC-T1 cells were exposed to various concentrations of ALA. As shown in Fig. 3, PpIX synthesis gradually increased with an increase in the ALA concentration and exposure time. The process of PpIX formation seemed to be saturated at ALA concentrations higher than 0.5 mM.

3.3. The effect of surfactant on the ALA uptake and PpIX formation

The effect of surfactants on ALA uptake and PpIX generation in HuCC-T1 cells was investigated. As shown in Fig. 4a, the amount of ALA in the cells was measured after 4 h of incubation in the presence of 0.25 mM ALA with or without surfactants. When surfactants were added to ALA treatment, ALA uptake increased in a dose-dependent manner. The ALA uptake ratio in the presence of surfactants (at 5 μg/ml PF68 or 100 μg/ml TW80) was about 60% higher than that in the absence of surfactants. This result is consistent with the increased PpIX fluorescence intensity, where the PpIX fluorescence also increased in the presence of surfactants (Fig. 4b). At a TW 80 concentration of 100 μg/ml (0.25 mM ALA), PpIX accumulation was approximately 60% higher than the control group. Furthermore, increased production of intracellular PpIX was observed under all surfactant treatment conditions.

3.4. Photodynamic therapy

The effect of ALA–PpIX concentration on photocytotoxicity was evaluated (Fig. 5). For PDT treatment, initially the HuCCT-1 cells were incubated for 4 h without ALA, and no significant phototoxicity was observed after irradiation under 630 nm light (data not shown). When cells were cultured for 18 h after irradiation with various doses of light, phototoxicity against HuCCT-1 cells increased with an increase in the surfactant and light doses. Photo-

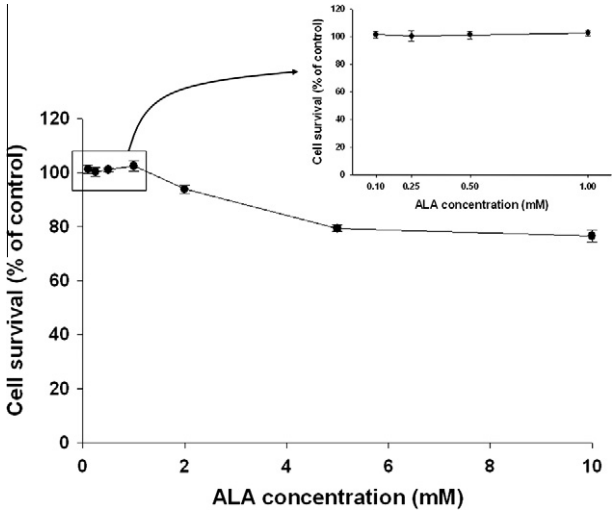

Fig. 1. Cytotoxicity of ALA. Human cholangiocarcinoma HuCC-T1 cells were incubated for 6 h in the presence of different amounts of ALA in the dark. Cell survival is expressed as the percentage of the control, which was not exposed to ALA, and determined by measuring MTT. Mean values and SD are presented (n = 8).

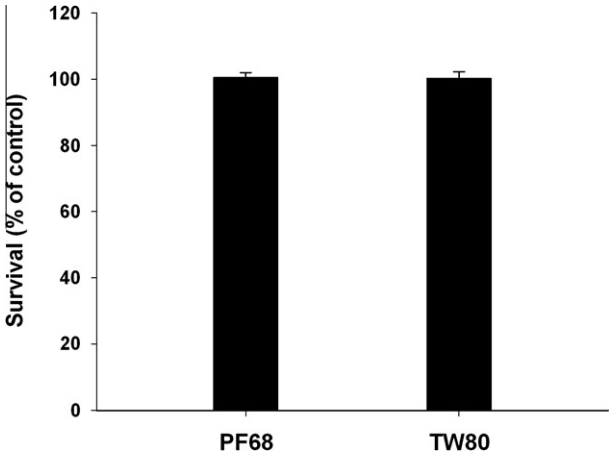

Fig. 2. Cytotoxicity of surfactants. Human cholangiocarcinoma HuCC-T1 cells were treated with surfactants (1 mg/ml) in serum-free culture medium for 6 h. Cell survival is expressed as the percentage of the control, which was not exposed to any surfactants, and determined by measuring MTT. Mean values and SD are presented (n = 8).

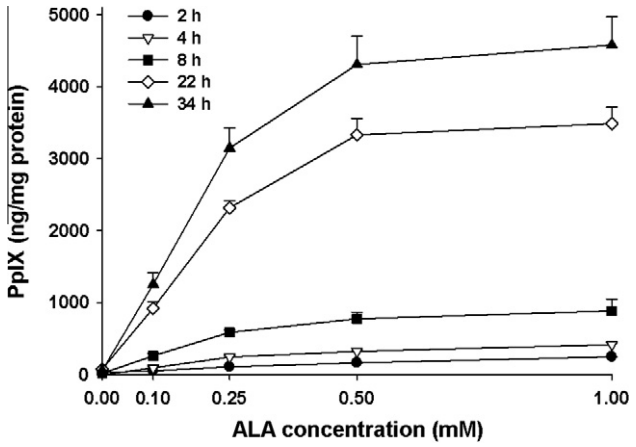

Fig. 3. PpIX synthesis from human cholangiocarcinoma HuCCT-1 cells incubated for set times with different concentrations of ALA in 96-well plates: incubation times were 2 h (circle), 4 h (inverted triangles), 8 h (squares), 22 h (diamonds), and 34 h (triangles). Intracellular PpIX was determined fluorometrically and reported as per mg of protein. Mean values and SD are presented ($n = 8$).

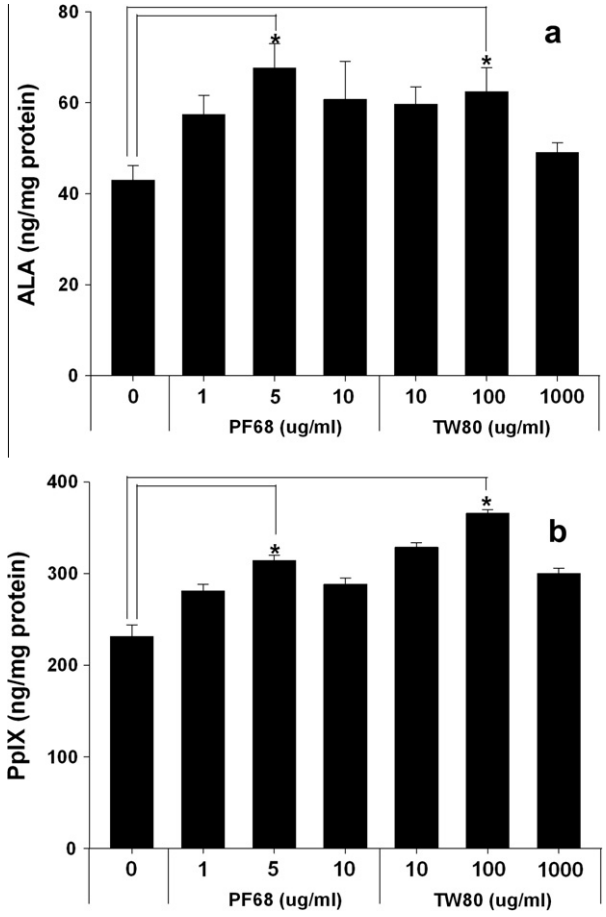

Fig. 4. The effect of surfactants on the (a) cellular uptake of ALA and (b) PpIX accumulation induced by 0.25 mM ALA. HuCC-T1 cells were exposed to ALA plus surfactants for 4 h. The PpIX concentration in the cells was corrected for protein content ($p < 0.05$).

toxicity increased by approximately 70% at an irradiation dose of 1.0 J/cm^2 (combined with 0.25 mM ALA and 100 μg/ml TW80) when compared with the treatment group with ALA only.

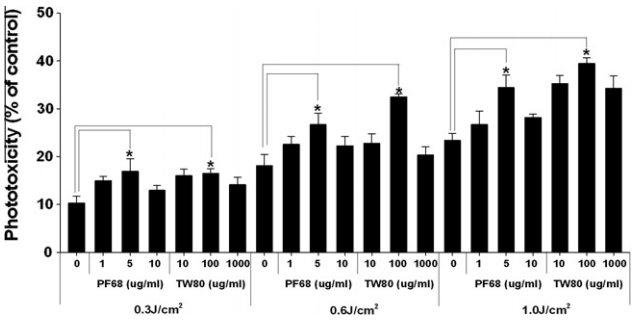

Fig. 5. The effect of surfactants on the phototoxicity against HuCC-T1 cells. The toxicity was measured using the MTT assay (expressed as a percentage of non-irradiated control samples); 2×10^4 HuCC-T1 cells were incubated for 4 h with 0.25 mM ALA in the presence of various concentrations of PF68 or TW80 and then irradiated under 0.3–1 J/cm^2 of red light. The results were obtained from an average of 8 wells ($p < 0.05$).

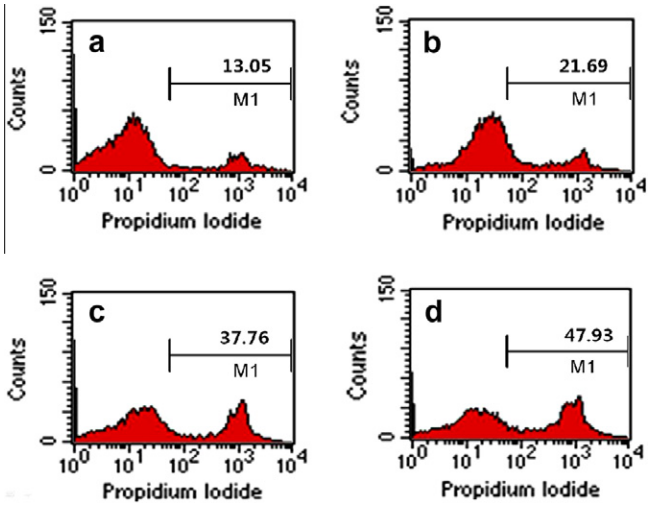

Fig. 6. Flow cytometric analysis of HuCC-T1: (a) cell-only control; (b) 0.25 mM ALA control; (c) with PF68 5 μg/ml; (d) with TW80 100 μg/ml. Cells were preincubated with 0.25 mM ALA and surfactants for 4 h. Twenty-four hour after exposure to a red light (1 J/cm^2), cells were stained with PI and measured using a flow cytometer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. In vitro photosensitizing efficacy

To detect whether phototoxicity against HuCC-T1 cells was increased via necrosis in PDT, the photo-cytotoxicity against the HuCC-T1 cell line of ALA with or without surfactants was evaluated (Fig. 6). Necrotic cells due to PDT were stained with PI and analyzed by flow cytometry. The necrotic cells were estimated to be around 21% and 48% for the absence (ALA treated only) and presence of 100 μg/ml TW80, respectively.

4. Discussion

Cholangiocarcinoma (CC) is one of the most difficult cancers to diagnose and treat [1,2]. Surgical operation is currently the most predominant method used to treat CC; however, most patients diagnosed with CC are in the latent CC state, and surgery is no longer a possibility. Since the benefit of additional radiotherapy or chemotherapy against non-resectable cholangiocarcinoma is still questionable, PDT is considered a promising palliative strategy to treat CC [19,20]. In this study, the HuCC-T1 cell line was used to

test the feasibility of 5-aminolevulinic acid (ALA)-based PDT for the treatment of CC.

A strong photosensitizer and appropriate light source are required to achieve successful PDT. To improve the PDT effect, the selection of a suitable photosensitizer is of primarily concern. The clinical use of PDT for the treatment of several tumors has demonstrated that this therapeutic approach has disadvantages such as long-lasting skin photosensitization induced by photosensitizers such as hematoporphyrin derivative [21,22]. ALA is a precursor of a photosensitizer and can be converted into strong photosensitizer, protoporphyrin IX (PpIX), via the heme pathway in cells. Moreover, PpIX and other intermediates that formed after ALA topical administration are rapidly eliminated from the body, limiting the risk of skin photosensitization to a few days [23]. The main drawback of the clinical application of ALA is its hydrophilicity, which results in poor cellular uptake [5]. Many researchers have attempted to develop methods to overcome this problem. For example, a ester unit was introduced to ALA to increase its lipophilicity [8,13,24,25]. Furthermore, an ALA dendrimer was also shown to be a feasible carrier to improve cellular uptake, and the ALA dendrimer was delivered to tumor cell via passive transport [26]. Bourre et al. reported that an uncharged ALA–peptide prodrug, phenylalanyl–ALA conjugate, has higher lipophilic properties, was chemically stable, and has improved ALA delivery [27].

On the other hand, a penetration enhancer has been shown to improve the cellular uptake of ALA. In fact, various enhancers have been shown to improve the penetration of ALA through the stratum corneum. Among the many types of penetration enhancers, including alcohols, sulfoxides, and fatty acids, surfactants are considered as a strong penetration enhancers. Most studies that evaluated the effects of surfactants have focused on the use of anionic and non-ionic surfactants, and non-ionic surfactants tend to be widely regarded as safe [10]. Non-ionic surfactants are believed to be potential delivery vehicles for enhancing peroral absorption of hydrophilic compounds such as protein and peptide drugs [28]. In addition, the balance between the hydrophilic and hydrophobic units of the surfactants can determine their application. Pluronic, which consists of ethylene oxide (EO) and propylene oxide (PO) segments, is a representative non-ionic surfactant [29]. Since pluronic F68 (*Mw* 8400) (PF68) had the highest hydrophilic–lipophilic balance (HLB) value, PF68 was regarded to be a more suitable hydrophilic agent than lipophilic agent. In addition, Tween 80 (TW80) has also been frequently used as an oil-in-water emulsion for the efficient delivery of hydrophilic drugs because it has a high HLB value (>10) [30]. Based on these attributes, we used PF68 and TW80 as enhancers to promote the cellular uptake of ALA. As shown in Fig. 4, PpIX accumulation was significantly enhanced by the addition of PF68 and TW80 in a concentration-dependent manner. However, ALA uptake and PpIX accumulation decreased when the PF68 and TW80 concentrations were higher than 10 μg/ml and 1000 μg/ml, respectively. Furthermore, similar results were observed in regard to photocytotoxicity (Fig. 5). ALA is extremely hydrophilic and cannot easily penetrate the cell membrane. Therefore, the addition of a surfactant to ALA affected its ability to penetrate the cell membrane. Most enhancers have been shown to interact with the intercellular lipid domain of the stratum corneum. Enhancers can modify the intercellular lipid domains to reduce the barrier resistance of the bilayer lipids [10]. A similar phenomenon could also occur for CC cells and, as a result, the level of ALA transported into the cell cytoplasm would increase when sufficient amounts of surfactants are present. Therefore, surfactant can increase ALA accumulation in the cells, which increases the PDT effect in HuCC-T1 cells relative to the control group. Hosmer et al. also investigated the effect of using TW80 as a transdermal delivery system for hydrophilic and hydrophobic drugs [31]. In this study, the transdermal flux of the hydrophilic drug, but not hydrophobic drug, was increased (2-fold) when microemulsions containing high amounts of surfactants were used, suggesting that increases in surfactants concentration can be used to enhance the transdermal delivery of hydrophilic compounds. We also obtained enhanced cellular uptake with the addition of PF68 and TW80, even though concentrations higher than 10 μg/ml PF68 and 1000 μg/ml TW80 did not improve the cellular uptake of ALA.

In general, the intracellular uptake of ALA and the concentration of PpIX interconversion increased according to the exposure time with ALA under a closed system. When compared with the *in vitro* cell culture, the bile duct of humans is an open system and the average basal flow of bile in humans is approximately 620 ml/day [32]. Therefore, hydrophilic substances, which are not fixed in drug carrying material such as polymer films or thermosensitive gels, are rapidly cleared from the bile duct. To maximize the PDT effect, ALA should be efficiently delivered to the tumor cells or tissues and converted to PpIX.

In this study, we showed that surfactants could enhance the uptake of ALA. When HuCC-T1 cells were treated with ALA at low concentration, the addition of non-ionic surfactants induced a higher ALA uptake and PpIX conversion ratio in tumor cells than ALA itself. Since non-ionic surfactants are known to be safe, they hold great promise for use as a vehicle for the delivery of ALA. Practically, the cytotoxicity of such compounds was negligible against cells (Fig. 2). In this study, we showed that low amounts of PF68 or TW80 (under 0.1%) are required to increase the cellular uptake of ALA and interconversion of PpIX. The phototoxicity of ALA plus non-ionic surfactants was also higher than that of ALA alone. Furthermore, ALA uptake, PpIX interconversion, and phototoxicity decreased when the concentrations were higher than 10 μg/ml PF68 and 1000 μg/ml TW80. Even though the reason is not clear at this moment, it indicates that there is an optimal surfactant concentration in regard to enhancing ALA uptake. Furthermore, we believe that non-ionic surfactants such as PF68 and TW80 can reduce the exposure time of ALA in ALA-based PDT and accelerate cellular uptake of ALA. This advantage of non-ionic surfactants is important to enhance ALA uptake because rapid absorption and cellular uptake of ALA is one of the most dominant factors in ALA-based PDT for cholangiocarcinoma. Consequently, non-ionic surfactants such as PF68 and TW80 are promising candidates for efficient delivery of ALA against cholangiocarcinoma.

5. Conclusion

We investigated the use of non-ionic surfactants, pluronic F68 (PF68) and Tween 80 (TW80), as an enhancer to improve the cellular uptake of ALA using human cholangiocarcinoma HuCC-T1 cells. Intracellular PpIX production following the administration of ALA was significantly increased in the presence of non-ionic surfactants. Furthermore, the increased intracellular PpIX fluorescence levels when ALA was combined with PF68 or TW80 were positively correlated with cellular phototoxicity, while the surfactants itself did not affect the viability of cells. Non-ionic surfactants are considered an effective vehicle for the delivery of the hydrophilic drug, ALA, against cholangiocarcinoma.

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